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Polyamine levels in various tissues of rats treated with 3-hydroxy-4-methoxycinnamic acid and 3,4-dimethoxycinnamic acid

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The effects of 3-hydroxy-4-methoxycinnamic acid (3H4MCA) and 3,4-dimethoxycinnamic acid (3,4DMCA) on body weight, organ weight, and the contents of putrescine, spermidine and spermine in 15 different tissues were examined in rats that had been given these compounds for 5 days. In 3H4MCA-treated rats, the weight of the spleen was significantly increased, while none of the other organs showed any significant changes. A diet containing either 3H4MCA or 3,4DMCA should not be taken by patients bearing cancers in the seminal vesicles, spleen or liver, and a diet containing 3,4DMCA should not be taken by patients bearing cancers in the testis, kidney, muscle, small intestine or brain (cortex) because of the significant increases in polyamines, which are associated with a risk of cancer growth, in these tissues. However, a diet containing 3H4MCA is recommended for the management of cancers in the skeletal muscle (femoral), tongue, small intestine (jejunum), stomach, lung and brain based on reductions in polyamines which stimulate tumor growth. A diet containing 3,4DMCA is also recommended for cancer in the prostate, thymus and stomach for the same reason. In addition, a synergic therapeutic effect for the treatment of cancers in these tissues may be anticipated by a combination of such a diet with anti-cancer drugs which reduce polyamine levels. The metastasis of cancers in these tissues may also be inhibited by the reduction of polyamines by these acids. The ratio of spermidine to spermine was significantly higher in the lung of 3H4MCA-treated rats, and lower in the seminal vesicle, thymus, kidney, heart, tongue, stomach and lung of 3,4DMCA-treated rats, than in control rats. The present experiment indicated that cancer patients should pay careful attention to endogenous polyamines in tissues bearing tumors induced by chemicals in ingesta and anti-cancer drugs, in addition to exogenous polyamines.

Key words: Caffeic acid derivative, cancer, polyamine, putrescine, spermidine, spermine.

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Introduction

The fact that tumors require naturally occurring polyamines (putrescine, spermidine and spermine) for growth has been demonstrated in many experiments. Intracellular polyamine concentrations are regulated by a combination of biosynthesis, retroconversion and transport from the dietary gastrointestinal tract,[1,2] and there is a strong positive correlation between intracellular polyamine concentrations and cell growth. For example, high concentrations are characteristic of rapidly growing cells, whereas slow-growing or quiescent cells have low or depleted polyamine levels.[3] In addition, the secondary endogenous polyamine content induced by chemical components in ingesta should also be considered. Huang *et al.* reported that caffeic acid and ferulic acid derivatives of cinnamic acid found in ingesta inhibited 12-*O*-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase activity in mouse epidermis and also inhibited 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in epidermal tumors initiated by 7,12-dimethylbenz[*a*]anthracene.[4] This fact strongly suggests that these acids inhibit tumor promotion by reducing polyamine levels through the inhibition of ornithine decarboxylase activity. Consequently, it is reasonable to surmise that the cinnamic acid derivatives 3-hydroxy-4-methoxycinnamic acid (3H4MCA) and 3,4-dimethoxycinnamic acid (3,4DMCA), which have a chemical structure quite similar to caffeic acid and ferulic acid, and which are found in some, if not all, foodstuffs, spices, flavorings and folk medicines of plant origin,[5–7] may possess anti-cancer activity based on their ability to reduce intracellular polyamine levels, as observed in the case of epidermal tumors. Cancer patients regularly consume

these acids in their diets, the folk medicines and foods given with anti-cancer drugs for therapy and physical recovery. We previously observed that anti-cancer drugs increased endogenous polyamine levels. The anti-cancer drugs ara-C,[8,9] 5-fluorouracil (5-FU),[9] adriamycin[10] and cisplatin,[10] which increase the intracellular polyamine content in many rat tissues, may lead to regrowth from trace cells in surviving and/or tumor cells that are tolerant to these drugs after the cessation of therapy.

In addition to these effects of polyamines, the reduction of polyamine levels in tissues enhances the chemotherapeutic effects of some anti-cancer drugs.[11] In fact, their anti-cancer efficacy is closely related to the polyamine level in the cancer-bearing tissues. The decrease in polyamine levels in cells enhances[12–17] or reduces[18–21] the anti-cancer efficacy of some anti-cancer drugs in specific tissues. Therefore, an analysis of the effects of 3H4MCA and 3,4DMCA, methoxy derivatives of caffeic acid which are commonly found in ingesta, on the polyamine content in tissues with various cell-cycle kinetics is necessary to identify whether these acids reduce or elevate polyamine levels. If these compounds reduce polyamine levels in specific tissues, they may be interesting as candidates and/or adjunct drugs for inhibiting metastases[22] and for treating cancer in these tissues in combination with drugs which work by reducing polyamines. Furthermore, an examination of the polyamine contents in the tissues of rats given these acids could be very useful in identifying what kind of folk medicines or foods should be taken as an important potential therapeutic tool for improving the management of cancer treatment based on the tumor-bearing tissues. In the present study, we used HPLC to determine the contents of individual polyamines and the total polyamine content in 15 tissues in rats given 3H4MCA and 3,4DMCA for five successive days. This is the first report that the content of endogenous polyamines induced by chemical components in the ingesta can play an important role in the management of cancer patients and in the choice of anti-cancer drugs.

Materials and methods

Chemicals

3H4MCA, 3,4DMCA and all of the polyamines and diamines used to prepare the standard solutions were purchased from Sigma (St Louis, MO). Potassium hydroxide, 2-mercaptoethanol, boric acid, *o*-phthalaldehyde, perchloric acid (60%), Brij-35, methanol and tri-sodium citrate dihydrate were obtained from Nacalai Tesque (Kyoto, Japan), and used without further purification.

Chromatographic analysis

Chromatographic analysis[23] was carried out using the JASCO analytical chromatographic system (JASCO, Tokyo, Japan) equipped with a JASCO 802-SC system controller, two JASCO 880-PU intelligent HPLC pumps, a JASCO 851-AS intelligent autosampler, a JASCO 860-CO column oven, a JASCO 821-FP intelligent spectrofluorometer, a JASCO 880-51 degasser and a JASCO 805-GI graphic integrator. For analytical procedures, we used a polyamine-pak column (35 × 6 mm), which was protected by a guard-pak column, both of which were made by JASCO. The flow rates were 0.75 ml/min for both the mobile-phase solution and the OPA reagent. The temperature of the column oven with the OPA reaction coil was kept at 70°C throughout the experiment. After post-column derivatization with OPA, the fluorescence intensity was measured with the intelligent spectrofluorometer (excitation at 340 nm, emission at 450 nm) and the amount of each polyamine was calculated from the peak area relative to the internal standard 1,6-diaminohexane.

Buffer and OPA reagent

The buffer solution for the elution system was prepared by dissolving 1.0 mol tri-sodium citrate dihydrate in water in a final volume of 1.0 l and the pH was adjusted to 5.3 with perchloric acid. This solution was filtered with a membrane filter (45 μm; Advantec, Tokyo, Japan) and degassed under a water aspirator at room temperature for 20 min.

The OPA–2-mercaptoethanol for the post-column derivatization procedure was prepared according to the method of Seiler and Knodgen[24] with minor modifications. Boric acid (24.7 g) and potassium hydroxide (23.0 g) were dissolved in water in a final volume of 1.0 l. After the addition of 2.0 ml of 2-mercaptoethanol to the mixture, the solution was filtered in the same manner as the buffer solution. This degassed solution was mixed with 2.0 ml of Brij 35 solution and 1.6 g of OPA dissolved in 10 ml of methanol. The OPA reagent, which was mixed with the solution for the HPLC system behind the polyamine-pak column, was allowed to react with each separated polyamine within the reaction coil with the column oven at 70°C.

Animals

Male Sprague-Dawley rats (35–38 days old, 170–180 g) were maintained on a 24 h light/dark cycle with light from 6.30 a.m. to 6.30 p.m. The conditions of animal housing were strictly controlled, and food and water were available *ad libitum*. Twenty one rats were divided into three equal groups: two experimental groups and a control group. The experimental groups received i.p. injections of 3H4MCA and 3,4DMCA in saline solution at 0.25 mmol/kg of body weight daily for 5 days. The control group received an injection of saline solution of the same volume. All of the rats were anesthetized with diethylether on the sixth day, and the tissues were immediately removed, weighed and kept in 2.0 ml of an aqueous 10% trichloroacetic acid (TCA) solution containing 0.1 mmol/l 1,4-diaminohexane as an internal standard in an ice bath. Each organ in the cold solution was homogenized with a homogenizer (TCU-2-110; Kinematica, Littau/Lucerne, Switzerland) and then centrifuged at 2500 r.p.m. for 15 min. The supernatants were washed twice with 5 ml of diethylether to eliminate the TCA in the water layer. The water layer was kept in a refrigerator below −20°C until measurement. The solution was passed through a millipore filter (45 μm; Cosmonice, Nacalai Tesque, Kyoto, Japan) and 10 μl of the filtrate was applied to HPLC by an autosampler.

Results

The effects of 3H4MCA and 3,4DMCA on body weight, the weights of the prostate, thymus, spleen, kidney, heart, seminal vesicles and testis, and the polyamine contents in these organs as well as in the liver, skeletal muscle (femoral), tongue, small intestine (jejunum), large intestine (rectum), stomach, lung and brain (cortex) were examined in rats that had been given the acids for 5 days at a dosage of 0.25 mmol/kg of body weight. The mean body weight did not show any statistically significant change in 3H4MCA-treated and 3,4DMCA-treated rats compared with control rats. The mean wet weights of the prostate, seminal vesicles, testis, thymus, spleen, kidney and heart in the 3H4MCA- and 3,4DMCA-treated rats, except for the spleen in 3H4MCA-treated rats, did not show a statistically significant change at this dosage, as shown in Table 1. The concentrations of the polyamines ranged

Table 1. The wet weights of the prostate, seminal vesicles, testis, thymus, spleen, kidney, heart and total polyamine contents (TP nmol/mg × wet weight) in these organs in rats given 3H4MCA or 3,4DMCA for 5 days (data represent the mean ± SE for each group)

Tissue	Drug	Weight (g)	TP/organ
Prostate	control	0.185 ± 0.025	2186.99 ± 406.79
	3H4MCA	0.192 ± 0.060	2273.07 ± 706.26
	3,4DMCA	0.178 ± 0.026	1747.49 ± 290.75
Seminal vesicles	control	0.463 ± 0.084	367.26 ± 75.34
	3H4MCA	0.433 ± 0.068	437.95 ± 36.29
	3,4DMCA	0.459 ± 0.047	444.50 ± 73.97
Testis	control	1.213 ± 0.053	593.29 ± 86.98
	3H4MCA	1.225 ± 0.042	660.06 ± 50.73
	3,4DMCA	1.175 ± 0.070	655.82 ± 93.94
Thymus	control	0.461 ± 0.087	1357.35 ± 236.75
	3H4MCA	0.476 ± 0.033	1447.52 ± 78.80
	3,4DMCA	0.486 ± 0.066	1208.06 ± 151.66
Spleen	control	0.560 ± 0.050	1031.85 ± 104.25
	3H4MCA	0.636 ± 0.073[a]	1269.12 ± 185.75[a]
	3,4DMCA	0.616 ± 0.102	1195.39 ± 140.44
Kidney	control	0.874 ± 0.070	835.62 ± 179.93
	3H4MCA	0.906 ± 0.065	888.90 ± 105.49
	3,4DMCA	0.886 ± 0.065	925.99 ± 108.49
Heart	control	0.701 ± 0.059	394.20 ± 77.44
	3H4MCA	0.695 ± 0.063	392.82 ± 35.85
	3,4DMCA	0.725 ± 0.048	449.79 ± 60.01

The statistical significance of differences was examined by Student's t-test. [a] $p < 0.05$.

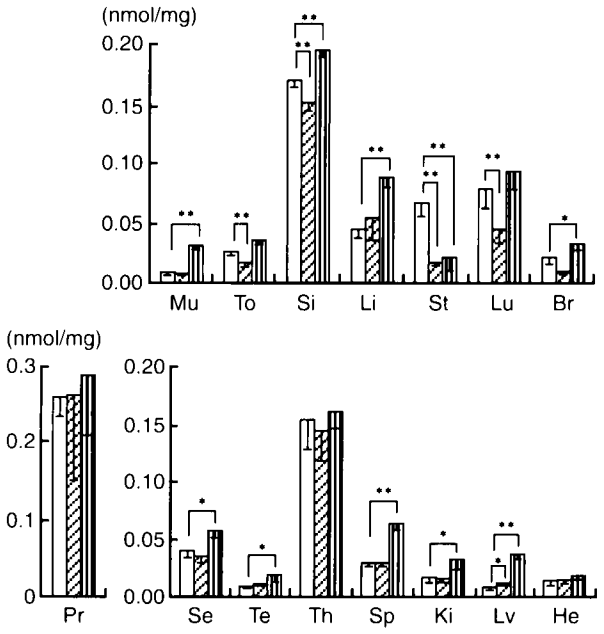

Figure 1. The content of putrescine per milligram of the wet weight of the prostate (Pr), seminal vesicles (Se), testis (Te), thymus (Th), spleen (Sp), kidney (Ki), liver (Lv), heart (He), skeletal muscle (Mu), tongue (To), small intestine (Si), large intestine (Li), lung (Lu), stomach (St) and brain (Br) of rats given 3H4MCA or 3,4DMCA for 5 days. Unshaded, control column; shaded, treated columns (right, 3,4DMCA; left, 3H4MCA). Columns represent the mean ± SE for each group. The statistical significance of differences was examined by Student's *t*-test. ** $p < 0.01$, * $p < 0.05$.

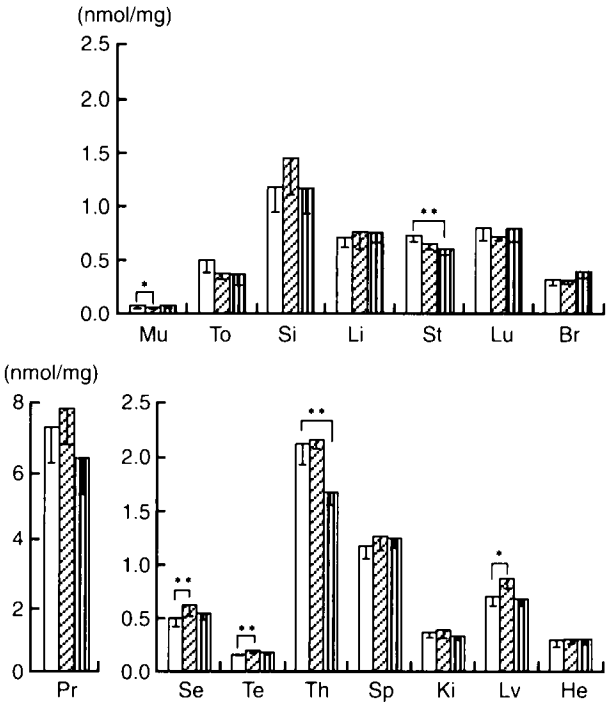

Figure 2. The content of spermidine per milligram of the wet weight of various tissues. Abbreviations and explanations are the same as in Fig. 1.

from about 0.02 to 0.3 for putrescine (Figure 1), 0.1 to 8.0 for spermidine (Figure 2), 0.2 to 4.2 nmol/mg for spermine (Figure 3) and 0.4 to 2.2 μmol/organ for total polyamines (Table 1) in each organ. The concentration of putrescine (Figure 1) ranged from about 1/10th (Mu/Mu) to 1/27th (Pr/Pr) that of spermidine (Figure 2) and from about 1/20th (Mu/Mu) to 1/13th (Pr/Pr) that of spermine (Figure 3). Putrescine in the liver and spermine in the seminal vesicles of rats treated with either 3H4MCA or 3,4DMCA, spermidine in the seminal vesicles, testis and liver, spermine in the spleen and liver of 3H4MCA-treated rats, and putrescine in the seminal vesicles, testis, spleen, kidney, muscle, small intestine, large intestine and brain, and spermine in the muscle and brain of 3,4DMCA-treated rats all showed statistically significant increases per milligram of wet weight compared with the values in control rats (Figures 1–3).

On the other hand, the putrescine (Figure 1) content in the stomach showed a significant decrease in both 3H4MCA- and 3,4DMCA-treated rats.

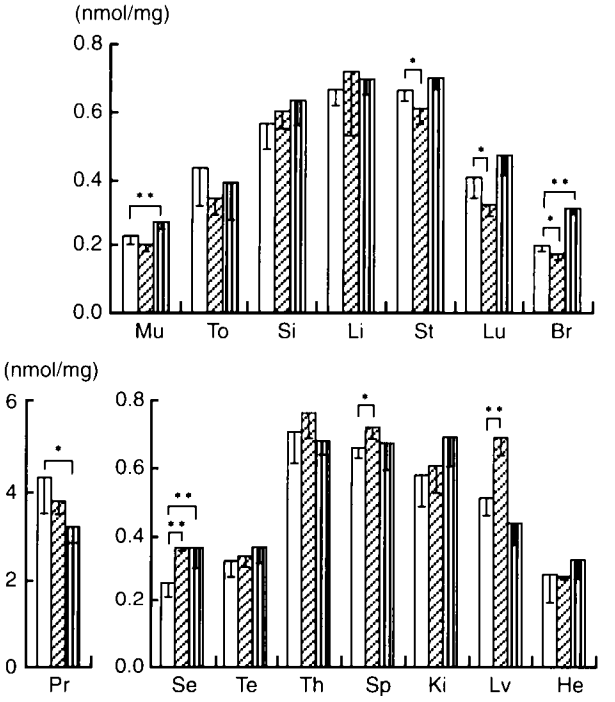

Figure 3. The content of spermine per milligram of the wet weight of various tissues. Abbreviations and explanations are the same as in Fig. 1.

Furthermore, putrescine in the tongue, small intestine and lung, spermidine in the muscle and spermine in the stomach, lung and brain of 3H4MCA-treated rats, and spermidine in the thymus and stomach, and spermine in the prostate of 3,4DMCA-treated rats all showed statistically significant decreases.

The spermidine/spermine ratio (Figure 4), which is considered to be an index of the growth rate, was significantly reduced in the seminal vesicles, thymus, kidney, heart, tongue, stomach and lung in 3,4DMCA-treated rats. However, it was higher than that of control rats in the lung of 3H4MCA-treated rats. There was no significant difference in this ratio between 3H4MCA- and 3,4DMCA-treated rats in any of the tissues tested.

Discussion

It is well known that cell proliferation is strictly dependent on polyamines and that a certain basal level of polyamines is required. Quemener *et al.* observed that polyamine deprivation by the combination of a polyamine-deficient diet with the polyamine oxidase inhibitor MDL72527, which prevents reutilization in the interconversion cycle, and α-difluoromethyl ornithine (DFMO), a specific and non-toxic irreversible inhibitor of ornithine decarboxylase which inhibits polyamine production, significantly enhanced the efficacy of chemotherapy without a concomitant increase in the toxic effect.[11,25] This strongly suggests that not only exogenous polyamines in folk medicines, foods such as spices, coloring agents, beverages and flavorings, but also endogenous secondary polyamines induced by chemical components in these materials can play an important role in tumor promotion and/or the management of cancer. Polyamine levels in tissues, which are important for cancer management, depend on various factors, including exogenous absorption from the gastrointestinal tract,[1,2] endogenous synthesis in cells induced by chemical components in the ingesta and by anti-cancer drugs, and endogenous reutilization through interconversion. In this study, we examined only one of these factors. An investigation of the effects of 3H4MCA and 3,4DMCA on the endogenous polyamine level induced in each tissue may be useful for managing cancer patients based on the polyamine level in cancer-bearing tissues, since these acids are widely distributed in ingesta. It is worth examining the effects of 3H4MCA and 3,4DMCA on the polyamine levels in 15 tissues because their chemical structures are quite similar to those of caffeic acid and ferulic acid, which are regularly eaten in foods and which have been shown to inhibit 12-*O*-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase activity, epidermal DNA synthesis and the promotion of skin tumors.[1] Without a significant change in the wet weight of the organs in rats given these compounds, significant changes in polyamine levels in some organs were observed. This means that these acids are interesting as possible anti-cancer drugs or their adjuncts.

The chemotherapeutic effects of some anti-cancer drugs, such as ethylmethansulfonate,[12] methylmethansulfonate,[12] arabinosyl cytosine,[13,14] anti-estrogen tamoxifen,[15] cyclophosphamide,[16] vindesine[17] and 1,3-bis-(2-chloroethyl)-1-nitrosourea[18] might be enhanced by the depletion of polyamines induced by these acids in ingesta, although the effects of these acids on the polyamine level in each tissue must be examined in full. For example, the antitumor effect of the anti-estrogen compound tamoxifen[15] on *N*-nitrosomethylurea-induced tumors is mediated through the induction of a depletion in polyamines, which may be under estrogenic control and may play an important role in mediating the effects of estrogens on tumor mitogenesis. In addition, the S

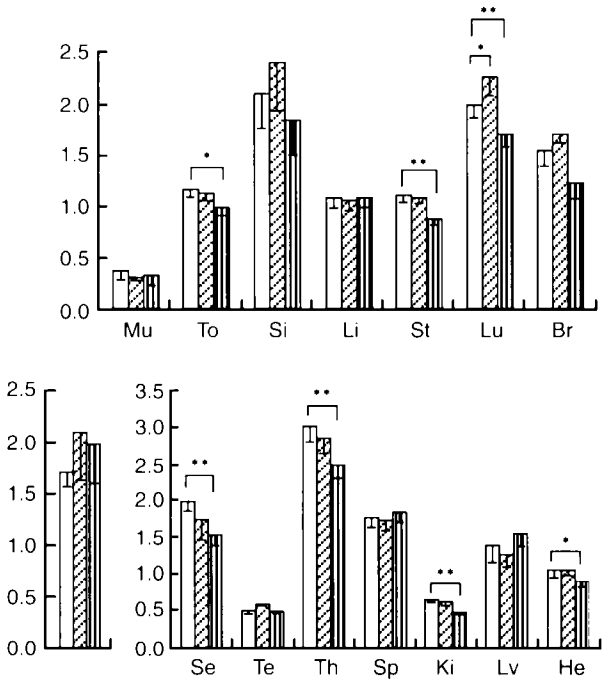

Figure 4. The ratio of spermidine/spermine in various tissues. Abbreviations and explanations are the same as in Fig. 1.

phase-specific drug arabinosyl cytosine[13,14] produced a preferential synergistic killing of tumor cells because transformed cells were arrested in S phase due to polyamine depletion in cells treated with DFMO. Furthermore, the sensitivity of HeLa cells to alkylating agents[12] increased because of an increase in altered chromatin structure and damage to cellular DNA induced by polyamine depletion in cells treated with DFMO. 3H4MCA decreased putrescine levels in the stomach, tongue, small intestine and lung, decreased spermidine in the skeletal muscle, and decreased spermine in the stomach, lung and brain, as shown in Figures 1–3. Furthermore, 3,4DMCA decreased putrescine levels in the stomach, decreased spermidine in the thymus and stomach, and decreased spermine in the prostate. These results indicate that the decrease in polyamines induced by these acid derivatives may be beneficial for treating cancers in these tissues in combination with anti-cancer drugs which work by reducing polyamine levels. Patients with tumors in these tissues should consume foods which contain these acids in combination with anti-cancer drugs which reduce polyamine levels. Thus, patients should pay considerable attention to their diets during and after cancer treatment.

However, a decrease in polyamines is not always beneficial for treating cancers in these tissues. DFMO-mediated polyamine depletion has been thought to lower the toxicity of cisplatin, based on the decrease in the formation of cross-linked DNA,[15,16] since a change in the DNA structure caused by polyamine depletion would make the cross-linking reaction with cisplatin mechanistically unfavorable. This result indicates that cisplatin would not be expected to have its best therapeutic effect in combination with these acids for the treatment of tumors in tissues in which these acids induce a decrease in the polyamine level.

On the other hand, the tumor regression brought about by anti-cancer drugs must compete with the tumor growth mediated by the increased polyamine content caused by these acids in ingesta. The polyamines deposited in the tissues by these acids might cause tumor cells to grow. The statistically significant increases in putrescine (Figure 1) in the liver and spermine (Figure 3) in the seminal vesicles of rats treated with either 3H4MCA or 3,4DMCA, in spermidine (Figure 2) in the seminal vesicles and liver, and in spermine in the spleen and liver of 3H4MCA-treated rats, and in putrescine in the seminal vesicles, testis, spleen, kidney, muscle, small intestine, large intestine and brain, in spermidine in testis, and in spermine in the muscle and brain of 3,4DMCA-treated rats clearly show that ingesta containing these acids should not be taken by patients with cancers in these tissues.

However, an increase in polyamines may also be beneficial for treating cancers in these tissues, considering the case of adriamycin. Polyamines are believed to reduce the cardiotoxicity of adriamycin (in the order: spermine, spermidine and putrescine), since they antagonize the binding of this drug to the inner membrane of heart mitochondria.[26] Furthermore, these polyamines in general seem to be effective in reducing free radical formation, which potentiates its cytotoxicity, based on findings that polyamines were able to significantly counteract paraquat-induced augmentation of lipid peroxidation and superoxide dismutase activity in the lung[27] and liver[28] of rats. Therefore, the cardiotoxicity of adriamycin may increase due to a decrease in polyamines in the heart in adriamycin-treated rats.

The ratio of spermidine/spermine, which is considered to be an index of growth suggesting hypertrophy,[29] was high in the lung of 3H4MCA-treated rats. However, the possibility of growth may be low because of the abnormal decrease in putrescine and spermine. On the other hand, this ratio in the seminal vesicles, thymus, kidney, heart, tongue, stomach and lung was lower than that in the control, while the levels of some polyamines were elevated in the seminal vesicles and kidney in 3,4DMCA-treated rats. Further study is needed to explain this contradictory relationship between this index and the polyamine level with respect to tumor growth.

Conclusion

Our findings regarding the effects of 3H4MCA and 3,4DMCA on the polyamine content in various tissues should be useful for deciding what kind of folk medicines or foods should be taken as an important potential therapeutic tool for improving the management of cancer treatment based on the tumor-bearing tissues. To identify the foods needed to achieve the best cancer management, the chemical components which induce endogenous polyamine content as well as the polyamine contents in ingesta must be studied in greater detail. While cancer patients may refuse anti-cancer drugs because of their severe toxicity, it is unlikely that they will refuse to eat. Accordingly, it is becoming increasing important that their diet should be viewed with respect to polyamine levels and induction.

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